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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
A61L 27/00, A61F 2/06	A1	WO 97/46266
		(43) International Publication Date: 11 December 1997 (11.12.97)

(21) International Application Number: PCT/US97/09633

(22) International Filing Date: 2 June 1997 (02.06.97)

(30) Priority Data:

08/660,653	3 June 1996 (03.06.96)	US
Not furnished	27 May 1997 (27.05.97)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

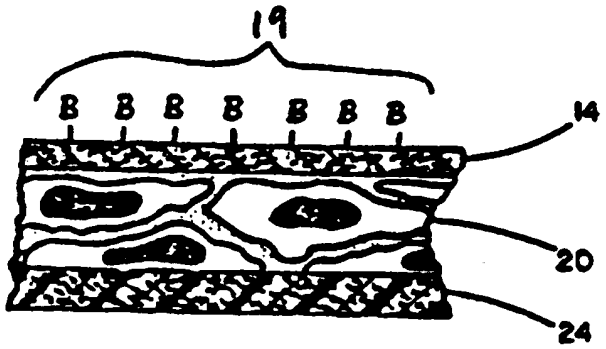
With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMPROVED BLOOD CONTACT SURFACES USING EXTRACELLULAR MATRIX SYNTHESIZED *IN VITRO* HAVING BIOACTIVE SPECIES IMMOBILIZED THERETO

(57) Abstract

This invention is directed to improved blood contact devices such as vascular prostheses rendered substantially nonthrombogenic through addition of a preserved layer of extracellular subendothelial matrix. The preserved subendothelial matrix layer, which serves as the blood interface of the device, is analogous to the subendothelial matrix layer beneath the endothelium of native vascular surfaces. The device consists of a permanent synthetic base material, preferably porous expanded polytetrafluoroethylene, on which this biologic layer of subendothelial matrix is grown *in situ*. The biologic layer is produced using *in vitro* tissue culture methods whereby living cells synthesize and deposit extracellular matrix components, after which the cells are killed and/or removed and the subendothelial matrix layer preserved before implantation. Bioactive species are then attached to the subendothelial matrix layer to enhance the patency performance of the matrix layer or as a mechanism through which a therapeutical treatment is administered to a patient. This invention results in vascular prostheses that are particularly useful for arterial bypass requiring a diameter of 6 mm of less.



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IMPROVED BLOOD CONTACT SURFACES USING EXTRACELLULAR MATRIX SYNTHESIZED *IN VITRO* HAVING BIOACTIVE SPECIES IMMOBILIZED THERETO

This application is a continuation-in-part of co-pending application Serial No. 08/660,653, filed June 3, 1996, which is a continuation-in-part of co-pending application Serial No. 08/424,839, filed April 19, 1995, which is a continuation-in-part of application of Serial No. 08/235,589, filed April 29, 1994, abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to improved blood contact surfaces for use in apparatus such as in artificial blood vessels and other implantable appliances, and methods for synthesizing the improved blood contact surfaces *in vitro*.

2. Description of Related Art

A common surgical practice in the treatment of occlusive atherosclerotic disease of peripheral arteries is to transplant a section of living vein, taken from the same patient, as a bypass around the occluded region of artery. Approximately one fourth of all patients requiring peripheral arterial bypass, however, have saphenous veins unsuitable for use because of varicosities, multibranching, or inadequate diameter. These patients then require some alternative material with which the bypass can be effected. In addition, the transplanted vein itself is often susceptible to the atherosclerotic disease process. This atherosclerotic process is similar to that of the artery but the vein is afflicted at an accelerated rate, frequently causing vein graft failure and necessitating an additional bypass. For these reasons, a need exists for a vascular substitute that would perform at least comparably to the autologous saphenous vein in the small diameter application.

In addition to autologous living tissues transferred from one location to another within the same individual, other biological materials have been used in

this application. These include treated donor allogeneic or xenogeneic tissue components, such as processed human umbilical vein, cryopreserved allogenic vein, or processed xenogeneic artery. Although living vessel transplants from one patient to another are employed, the foreign vessel typically dies following transplant.

In addition to tissues of biological origin, synthetic materials are also commonly used to replace blood vessels. Synthetic vascular grafts have been used successfully since the 1950's to replace large vessels such as the aorta or iliac arteries. The principal synthetics used for grafting include polyethylene terephthalate and expanded polytetrafluoroethylene (ePTFE), although other materials utilized include polypropylene, polyurethane, and polydimethyl siloxane. Both porous and nonporous constructions of these polymers have been used.

Although both biologic and synthetic materials have been used with good success in some applications, such as bypasses from the femoral artery to the segment of the popliteal artery above the knee, both synthetic and biologic materials have been shown to thrombose much more frequently than the autologous saphenous vein when used for small diameter bypasses such as the coronary or below knee arteries. This performance difference limits the usefulness of such vascular replacements in these more demanding applications.

Natural blood contact surfaces, such as those found within blood vessels, have inherent mechanisms to prevent thrombosis during normal passage of blood along the surface. In the case of a mammalian artery, the immediate blood contact surface consists of a layer of endothelial cells that is nonthrombogenic. Immediately external to the endothelial cell layer is the remainder of the intima: a subendothelial matrix layer consisting of basement membrane and an underlying layer of glycoprotein-bearing extracellular matrix, and the internal elastic lamina. Surrounding the intima layer is the multilaminate media structure containing smooth muscle cells and elastin, and surrounding this, the most external layer,

comprised of fibroblasts and connective tissue, the adventitia. As is explained in greater detail below, it is generally accepted that the subendothelial layer and media are thrombogenic in nature in order to maintain hemostasis when the vascular system is injured. See for example: J. A. Madri et al., "The Collagenous Components of the Subendothelium," Lab. Invest. 43:303-15 (1980); and T. Matsuda et al., "A Hybrid Artificial Vascular Graft Based Upon an Organ Reconstruction Model: Significance and Design Criteria of an Artificial Basement Membrane," ASAIO Transactions 34:640-43 (1988).

Knowledge of the detailed mechanisms by which natural vessels maintain patency has been very limited. The predominate theory relating to the effective function of normal vasculature is based upon the necessity of a healthy, intact, vascular endothelium to serve as the blood interface. When the endothelial lining is removed, thrombosis of the vessel is a frequent occurrence. Supporting this theory, in part, are numerous experiments showing the endothelium to have a unique clot inhibiting effect on blood with which it is in direct contact. Endothelial cells have been further shown to synthesize or bind a number of substances with coagulation inhibiting or fibrinolytic function including heparan sulfate/antithrombin III, dermatan sulfate/heparin cofactor II, thrombomodulin/protein C/protein S, prostacyclin and tissue-type plasminogen activator. Thus, based on these experimental observations, and the blood contacting location of the endothelium in the vascular system, it has been widely accepted that the endothelium is primarily, if not wholly, responsible for the antithrombotic behavior of blood vessels. See for example, R. G. Petty et al., "Endothelium-the Axis of Vascular Health and Disease," J. Royal Coll. Phys. London 23:92-102 (1989); and R. E. Scharf et al., "Thrombosis and Atherosclerosis: Regulatory Role of Interactions Among Blood Components and Endothelium," Blut 51:31-44 (1987).

In support of this concept, numerous experiments investigating the reactivity of blood to non-endothelialized vessels have been reported suggesting

that the subendothelium and underlying structures are thrombogenic, particularly with respect to platelet adhesion and degranulation. These observations come from both *in vivo* and *in vitro* thrombosis assays.

In addition to the immediate subendothelial matrix layer, smooth muscle cells in the deeper media layer are generally considered to be thrombogenic as well. See for example, S. M. Schwartz et al., "The Aortic Intima: II. Repair of the Aortic Lining After Mechanical Denudation," Am. J. Pathol. 81:15-42 (1975); J. J. Zwaginga et al., "Thrombogenicity of Vascular Cells: Comparison between Endothelial Cells Isolated from Different Sources and Smooth Muscle Cells and Fibroblasts," Arteriosclerosis 10:437-48 (1990). Evidence for this conclusion comes from studies where the addition of plasma to a culture of subendothelial cells including vascular smooth muscle cells has been shown to cause rapid, massive coagulation. In contrast, clotting was inhibited when the experiment was repeated with endothelial cell cultures, again emphasizing the nonthrombogenic nature of endothelial cells. See for example, P. Colburn and V. Buonassisi, "Anti-clotting Activity of Endothelial Cell Cultures and Heparan Sulfate Proteoglycans," Biochem. Biophys. Res. Comm. 104:220-27 (1982); and I. Vlodavsky et al., "Platelet Interaction with the Extracellular Matrix Produced by Cultured Endothelial Cells: A Model to Study the Thrombogenicity of Isolated Subendothelial Basal Lamina," Thromb. Res. 28:179-91 (1982).

The widely accepted interpretation of these observations of natural vessel function is that the endothelial cell lining of the vasculature is responsible for antithrombotic behavior and that the subendothelial layers as well as the smooth muscle cells found beneath the endothelium are thrombogenic so that hemostasis will result in the event of vessel disruption.

Not surprisingly, in an effort to adapt the antithrombotic function of the endothelium to synthetic surfaces, specifically vascular grafts, most prior art references that utilize biological elements are directed toward providing a surface that will support an endothelial cell lining. For example, U.S. Patents 4,539,716

and 4,546,500 issued to Bell disclose a method of constructing a living tubular prosthesis using a collagen gel to which cells are added. The cells serve as a contractile agent and are specified to be fibroblast cells, smooth muscle cells, or platelets. For an artery replacement, these patents specify the use of endothelium as the most internal layer, smooth muscle cells as the medial layer and a third layer cast of collagen and fibroblast cells. The endothelial cells employed are of unspecified origin.

In addition, U.S. Patents 4,804,381 and 4,804,382 issued to Turina et al. describe a synthetic arterial vessel made with a microporous or semipermeable membrane, lined on the luminal side with a continuous layer of living endothelial cells to provide the blood interface, and coated on the outside with layers of smooth muscle cells to increase the viability of the live cells on the lumen and to impart elasticity.

A number of other U.S. Patents including, for example, 4,883,755 to Carabasi et al., 4,960,423 to Smith, and 5,037,378 to Muller et al., also describe means by which living endothelial cell coverage of vascular interfaces can be accomplished to produce antithrombogenicity. These approaches include endothelial cell sodding, the use of elastin-derived peptides, and simple physical means of applying endothelial cells to graft surfaces, respectively.

A structure similar to that of Bell, above, is taught by H. Miwa et al., "Development of a Hierarchically Structured Hybrid Vascular Graft Biomimicking Natural Arteries," ASAIO Journal 39:M273-77 (1993). In this case, smooth muscle cells are layered over a DACRON® graft in an applied artificial matrix of collagen type I and dermatan sulfate glycosaminoglycan. A layer of endothelial cells is then grown on the artificial matrix to serve as the blood contact surface.

Another approach to endothelialization of vascular grafts is disclosed by X. Yue et al., "Smooth Muscle Cell Seeding in Biodegradable Grafts in Rats: A New Method to Enhance the Process of Arterial Wall Regeneration," Surgery 103:206-12 (1988). These authors employ pre-clotted microporous,

biodegradable, polyurethane vascular grafts seeded with nonautologous rat smooth muscle cells prior to implantation for use as the replacement of the abdominal aortas of living rats. This study attempts to generate a "neomedia" to strengthen a structure that would otherwise be mechanically insufficient following the resorption of the biodegradable graft material. The smooth muscle cell layer provides a surface upon which the host's natural endothelial cells could spontaneously regenerate and cover the graft surface.

In another study, for example, A. Schneider et al. used corneal endothelial cells to produce extracellular matrix on ePTFE vascular grafts. (A. Schneider et al., "An Improved Method of Endothelial Seeding on Small Caliber Prosthetic Vascular Grafts Coated with Natural Extracellular Matrix," Clin. Mat. 13:51-55 (1993)) After production of an extracellular matrix, these original cells were then removed using Triton X-100 and NH_4OH, and the tubes were seeded again with bovine aortic endothelium. This approach showed that endothelium could be successfully grown on the extracellular matrix lining the ePTFE grafts, but no implant studies were performed, however.

In all of the above described prior art references, the efforts are directed at achieving a living endothelial cell lining to provide nonthrombogenic function. Despite the above described efforts, substantial deficiencies still exist. In small diameter applications or grafts used in sensitive areas, for example, even limited thrombus generation is a very serious concern. It should also be noted that the *in vivo* performance of the above endothelial cell-coated grafts has been highly variable, without a clear demonstration of enhanced patency over existing grafts.

Accordingly, it is a primary purpose of the present invention to provide an improved blood contact surface that is less thrombogenic than existing artificial surfaces and structures.

It is another purpose of the present invention to provide an artificial blood contact surface that can be readily manufactured and used.

It is a further purpose of the present invention to employ a previously

unrecognized mechanism governing the interaction between blood and natural blood vessel structures and to produce an improved blood contact device and methods for making and using them.

These and other purposes of the present invention will become evident from review of the following specification.

SUMMARY OF THE INVENTION

The present invention is an improved blood contact surface suitable for use in a variety of appliances, including artificial blood vessels and other implantable blood contact devices.

This invention employs an extracellular matrix synthesized *in situ* by selected cells on synthetic surfaces. Surprisingly, this extracellular matrix emulates the antithrombotic properties of natural vessels and organs. The matrix is preferably produced in cell culture using cells derived from vascular tissue.

In a first step of the process, smooth muscle cells (SMCs) obtained from autologous, allogeneic, or xenogeneic sources are grown under mitogenic conditions, initially in culture dishes, then subsequently on the intended blood contact surface of a prosthesis until confluent coverage of the synthetic base material surface is largely achieved.

In a second step, the surface of the smooth muscle cell layer is seeded with endothelial cells derived from the surface of blood vessels. The two cell types are grown in co-culture on the surface of the prosthetic under growth conditions until an extracellular matrix is produced between the layers. This matrix is the analogue of the subendothelial matrix found in normal arterial vessels.

In a third step, the endothelial cells are removed so as to expose the subendothelial matrix overlying the SMCs. This is a key inventive feature as the prior art teaches directly away from this approach. As described in the background section above, the prior art teaches that endothelial cells are the appropriate blood

contact surface. Thus, a key element of the present invention is a direct blood contact surface absent of living cells, especially endothelial cells. A further key element is the *in vitro* production of a subendothelial matrix analogue that results in an antithrombotic, non-immunogenic, vascular surface of desired size and shape. In the final steps, the subendothelial matrix may be preserved and sterilized using an appropriate fixative agent and a sterilizing agent, if necessary. Subsequently, useful biological compounds and/or biological entities are attached to the subendothelial matrix layer through chemically reactive groups on the matrix. Collectively, biologically useful compounds and biological entities are referred to herein as "bioactive species." Attachment of bioactive species to the subendothelial matrix layer permits the layer to be modified to further improve patency performance of the matrix layer or as a mechanism through which a therapeutical treatment is administered to a patient. The bioactive species may be permanently attached to the subendothelial matrix layer or temporarily attached thereto.

It will be recognized that artificial blood vessels are only one example of the high demand blood interface applications of this invention. Other possible applications include heart valves, artificial hearts, other artificial organs such as implantable artificial kidneys, and other direct blood contact appliances.

In one embodiment, the present invention is a blood contact surface comprising a synthetic base material, a first layer of cells attached to the synthetic base material, and a second layer attached to the first layer wherein the second layer comprises subendothelial matrix substantially free of endothelial cells, wherein the subendothelial matrix layer serves as a direct blood contact surface, and wherein bioactive species are attached to the subendothelial matrix layer.

DESCRIPTION OF THE DRAWINGS

The operation of the present invention should become apparent from the

following description when considered in conjunction with the accompanying drawings.

Figure 1 is a perspective sectional view of a mammalian artery (10).

Figure 2 is a sectional view through the longitudinal axis of a mammalian artery (10).

Figure 3 is a cross-sectional view of a layer of smooth muscle cells (20) applied to a synthetic base material (24).

Figure 4 is a cross-sectional view of a layer of endothelial cells (12) applied directly to a layer of smooth muscle cells (20) lying on a synthetic base material (24).

Figure 5 is a cross-sectional view of the structure shown in Figure 4 once a subendothelial matrix (14) has been generated between the endothelial cells (12) and the smooth muscle cells (20).

Figure 6 is a cross-sectional view of one embodiment of the present invention wherein the endothelial cell layer (12) has been removed from the structure of Figure 5.

Figure 6A is a cross-sectional view of one embodiment of the present invention wherein the endothelial cell layer (12) has been removed from the structure of Figure 5 and bioactive species (19) attached to the subendothelial matrix layer (14).

Figure 6B is a cross-sectional view of one embodiment of the present invention wherein the endothelial cell layer (12) has been removed from the structure of Figure 5 and bioactive species (19) attached to the subendothelial matrix layer (14) through a spacer compound (22).

Figure 7 is a view of a tubular embodiment (26) of the present invention illustrated in Figure 6.

Figure 8 is a view of one embodiment of the present invention wherein the structure shown in Figure 6 is grown on a flat sheet of synthetic base material (24).

Figure 9 is a view of a heart valve appliance (30) employing the

subendothelial matrix (14) of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to improved blood contact surfaces, such as those used in artificial blood vessels and other blood contact appliances.

By way of introduction, Figures 1 and 2 illustrate the structure of a typical mammalian artery (10). The artery (10) structure consists of an intima layer having an innermost layer of endothelial cells (12), a subendothelial matrix layer (14) consisting of basement membrane and other extracellular matrix components, and an internal elastic lamina layer (16). External to the intima is a media layer (18) composed of smooth muscle cells (SMC) (20), and finally a fibrous connective tissue or adventitia layer (22).

Figure 6A illustrates an embodiment of the present invention wherein bioactive species (19) are attached to the subendothelial matrix layer (14) through chemically reactive functional groups of the matrix. Similarly, Figure 6B illustrates an embodiment of the present invention wherein bioactive species (19) are attached to the subendothelial matrix layer (14) through an intermediate spacer compound (22).

As has been explained in the background section above, during normal blood flow the endothelial layer of cells (12) serves as the blood contact surface and, accordingly, must be nonthrombogenic. By contrast, when the blood vessel has been damaged, it is important that thrombosis occurs to prevent blood leakage from the vessel. In this respect, it has been generally accepted that the structures under the endothelial cell layer are thrombogenic in order to promote clotting wherever the endothelial layer has been compromised.

In contrast to the previous body of evidence in the prior art regarding blood vessel patency, it has been discovered that there is a mechanism independent of the living endothelium that contributes to the antithrombotic behavior of the vessel.

Surprisingly, the location of this mechanism is not at the immediate blood contact surface of a native vessel but internal to the endothelial layer. This antithrombotic mechanism is dependent upon the subendothelial extracellular matrix previously reported in the prior art to be thrombogenic.

The present invention uses an analogue of the subendothelial matrix, synthesized with living vascular cells *in vitro*, to provide a substantially nonthrombogenic blood interface surface for synthetic materials. This subendothelial matrix is preferably produced by first culturing a substrate cell layer on a synthetic base material for a period of time. It is preferred that the substrate cell layer be formed with smooth muscle cells (SMCs), and most preferably vascular smooth muscle cells (VSMCs). Once the substrate cell layer is created, it is seeded with endothelial cells (ECs), preferably of vascular origin, to re-establish a conventional vascular cell relationship. Following a culture period of sufficient time to allow both the SMCs and ECs to synthesize an *in situ* subendothelium, the ECs are specifically removed in such a manner as to leave the subendothelial matrix layer, substantially free of endothelial cells, overlying the SMCs on a synthetic base material. The goal of this treatment is to remove the endothelial cells while preserving the anti-thrombotic properties of the underlying extracellular matrix. It should be recognized that many of the methods commonly used to remove cells from culture surfaces will disrupt the layer of interest. Processes with the potential to alter the subendothelial matrix layer include exposure to enzymes such as trypsin or dispase, or exposure to detergents such as Triton X-100 or sodium dodecyl sulfate. The composite graft may then be stabilized by a fixation step, preferably using glutaraldehyde, to minimize immunogenicity and preserve the subendothelial matrix layer that will serve as a direct blood contact surface of the graft. The term "preserved subendothelial matrix layer," in the instant specification, refers to a subendothelial matrix layer, that has been treated with a fixative solution, such as glutaraldehyde, to chemically stabilize the matrix and thereby preserve the subendothelial matrix layer as a direct

utilizing spacer arms allow the introduction of a tether between the subendothelial matrix and the bioactive species.

Bioactive species are attached to a non-preserved subendothelial matrix layer by activating its constituent protein and proteoglycan components using suitable activation protocols; the activated matrix constituents may then be reacted with bioactive species to graft the bioactive species directly onto the matrix. For example, the unpreserved graft is immersed in a buffered solution of about 1 - 10% EDC, about pH 5 - 10, for about 0.5 - 6 hr at about 4 - 25°C. The activated graft is then rinsed in saline, and then immersed in a buffered solution of bioactive species at a concentration of about 0.01 - 10%, for about 24 hours at about 4 - 25°C. Following grafting of the bioactive species onto the activated subendothelial matrix, the graft is rinsed in saline. Alternatively, the EDC and the bioactive species may both be present, to allow activation of and grafting to the subendothelial matrix to proceed simultaneously. Activation protocols utilizing spacer arms allow the introduction of a tether between the matrix and the bioactive species.

At this stage, a number of options exist for use of the subendothelial matrix having bioactive species attached thereto. Given the nonthrombogenic nature of the subendothelial matrix (14), it is possible to implant the tubular graft structure substantially. Alternatively, the subendothelial matrix layer having bioactive species attached thereto may be removed and directly applied to a damaged vessel *in vivo*. Further, in the case of bioactive species in the form of autologous cells, the graft may be implanted with the cells in a viable state.

It is also possible to produce the subendothelial matrix layer through a variety of other methods. One suitable method, for example, involves using mixed culture seeding in which both ECs and SMCs are combined in ratios of 1:10 to 1:1 (EC:SMC) and both cell types are seeded onto the synthetic base material simultaneously. Once placed into culture, the ECs will form a confluent monolayer on the luminal surface thereby reestablishing the normal EC and SMC

blood contact surface.

While the following examples are specific for vascular graft prosthetics, it should be recognized that artificial blood vessels are only one example of the high demand blood interface applications of this invention. Other applications to which this invention may be applied include heart valves, artificial hearts, and artificial organs such as implantable artificial kidneys among others.

As is explained in greater detail below, it has been shown that the use of the subendothelial matrix as a direct blood contact surface resolves many of the previous deficiencies of the prior art in creating substantially nonthrombogenic blood contact surfaces. First, the use of a natural subendothelial matrix layer is less prone to thrombus generation and other problems than presently available prosthetic grafts. This allows such grafts to be used in small diameter grafts and other applications that are particularly prone to thrombotic failure. Second, in artificial graft production, the provision of a natural subendothelial matrix layer as a substantially nonthrombogenic surface eliminates the need to provide a prosthesis having an endothelialized surface.

Additional utility is conferred on a vascular graft of the present invention when bioactive species are attached to the subendothelial matrix layer. Bioactive species include enzymes, organic catalysts, ribozymes, organometallics, proteins, glycoproteins, peptides, polyamino acids, antibodies, nucleic acids, steroidal molecules, antibiotics, antimycotics, cytokines, carbohydrates, proteoglycans, oleophobics, lipids, pharmaceuticals, and therapeutics, for example. Cells, such as, mammalian cells, reptilian cells, amphibian cells, avian cells, insect cells, planktonic cells, cells from non-mammalian marine vertebrates and invertebrates, plant cells, microbial cells, protists, genetically engineered cells, and organelles, such as mitochondria, are also bioactive species. In addition, non-cellular biological entities, such as viruses, and virenos are considered bioactive species. As used herein, the term "attach" and its derivatives refer to ligand/receptor interactions, covalent bonding, hydrogen bonding, or ionic bonding of a bioactive

species to a subendothelial matrix layer. A subendothelial matrix layer can comprise a preserved matrix as defined above or a non-preserved matrix substantially free of endothelial cells. The principle determinant of whether the bioactive species is attached to the subendothelial matrix layer before or after preservation of the matrix layer is the stability of a bioactive species to the procedure used to preserve the subendothelial matrix layer.

Preparation and Use of *In Vitro* Subendothelial Matrix

The source of subendothelial matrix for use in the present invention is based upon *in vitro* tissue culture methods. While the precise constituents producing antithrombogenicity are not yet fully understood, techniques have been developed that hold promise for producing the matrix on a large scale basis.

The natural generation of subendothelial matrix appears to be the result of interaction between the endothelial cells (12) and the smooth muscle cells (20). In fact, it has been determined that a particularly effective blood contact surface can be generated *in vitro* by co-culturing a layer of smooth muscle cells with a layer of endothelial cells in tissue culture and allowing the co-cultured layers to form a subendothelial matrix layer between them. The steps of one process in this regard are illustrated in Figures 3 through 6.

The initial step in this process involves the preparation of a synthetic base material to support the smooth muscle cell layer. The preferred base material consists of a synthetic porous, expanded polytetrafluoroethylene (ePTFE) graft material, such as those commercially available from W. L. Gore & Associates, Inc., Flagstaff, AZ, under the designation GORE-TEX® Vascular Graft. The 4 mm internal diameter vascular grafts used in the following description are commercially available product obtained from this source. The 2.5 mm internal diameter ePTFE tubing used in following description were constructed from CD 123 fine powder PTFE resin (ICI Americas) as taught in U.S. Patent 3,953,566 to

Gore, which is incorporated herein by reference. The tubes were expanded by stretching to produce a mean fibril length of 28 μm. A fibril length of less than about 60 μm is preferred for this application. The finished tubes had an internal diameter of about 2.5 mm and a wall thickness of about 0.33 mm.

The fibril length of the porous ePTFE tubes produced as above is defined herein as the average of ten measurements between nodes connected by fibrils in the direction of expansion. Ten measurements are made in the following manner. First, a photomicrograph is made of a representative portion of the sample surface, of adequate magnification to show at least five sequential fibrils within the length of the photograph. Two parallel lines are drawn across the length of the photomicrograph so as to divide the photograph into three equal areas, with the lines being drawn in the direction of expansion and parallel to the direction of orientation of the fibrils. Measuring from left to right, five measurements of fibril length are made along the top line in the photograph beginning with the first node to intersect the line near the left edge of the photograph and continuing with consecutive nodes intersecting the line. Five more measurements are made along the other line from right to left beginning with the first node to intersect the line on the right hand side of the photograph. The ten measurements obtained by this method are averaged to obtain the fibril length of the material.

Other suitable synthetic base materials may include but not be limited to the following: porous PTFE, polyethylene terephthalate, polypropylene, polyurethane and polydimethyl siloxane.

The synthetic base material suitable for the current invention was further prepared in the following manner. Commercially available 4 mm diameter GORE-TEX® Vascular Grafts (W. L. Gore & Associates, Inc., Flagstaff, AZ) and ePTFE tubing measuring 2.5 mm inside diameter were cut to 7 cm lengths and syringe fittings were tied to both the proximal and distal ends of the grafts. Each graft was then mounted in a stainless steel wire holder and a plug inserted into the connector at the distal end of the graft. After steam sterilization, the grafts were prepared for

cell-seeding by wetting the normally hydrophobic ePTFE with 100% ethanol. The ethanol in the graft interstices was displaced with about 80-100 ml of Hanks' Balanced Salt Solution (HBSS) (Gibco BRL, Grand Island, NY) using a syringe attached to the proximal connector. Wetted grafts were stored in HBSS until used for cell-seeding with smooth muscle cells.

Once the base material tube has been prepared, SMCs (20) are applied to the luminal surface of the synthetic base material (24) producing a structure resembling that shown in Figure 3. The preferred method is the use of positive pressure to force the SMCGM through the graft wall depositing SMCs onto the base material luminal surface. Other suitable means for applying the SMCs to the base material may include, but not be limited to: filling the base material tube lumen with a SMC suspension followed by a series of graft rotations to allow the SMCs to settle onto the surface uniformly; using negative pressure to draw the SMCs onto the substrate; and using chemotactic agents.

In several experiments, for example, the VSMCs were procured and applied to the synthetic base material in the following manner. Vascular SMCs were isolated by placing 3-4 cm segments of carotid or femoral arteries obtained from greyhound dogs into a tube containing cold, sterile Medium 199 and 50 μg/ml gentamicin (Gibco BRL). In a laminar flow hood, the artery segment was slit longitudinally and the endothelial cells were removed by first rubbing the luminal surface with a sterile paper towel followed by scraping with a #10 scalpel blade. Thin strips of arterial media were peeled up with forceps and pooled into a puddle of HBSS in a sterile Petri dish. The strips were then placed into 25 cm^2 tissue culture flasks containing 1.5 ml Smooth Muscle Cell Growth Medium (SMCGM; 43% Dulbecco's Modified Eagle Medium (DMEM); 43% Medium 199; 13% fetal bovine serum; 2 mM glutamine; 15 units/ml heparin; 23 μg/ml gentamicin; and 12.5 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA)). Culture medium in the flasks was replaced when significant outgrowth of cells from the tissue pieces was observed. Cells were then

fed 3-5 ml twice weekly, depending on the number of cells in the T-25 flask. Cells were generally passaged when about 60-90% confluent, and were usually split about 1:4. Smooth muscle cell type was confirmed by morphological criteria, positive staining for alpha smooth muscle cell actin, and lack of uptake of acetylated low density lipoprotein which would indicate endothelial cell (EC) contamination.

For graft-seeding purposes, subconfluent VSMC cultures (about passages 3-15) were rinsed briefly with calcium-magnesium-free-HBSS (CMF-HBSS) and washed in CMF-HBSS for about 3 to about 5 minutes. Cells were harvested using trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA) to release cells from the flask, followed by trypsin neutralization with SMCGM. Cells were pelleted in a centrifuge at about 300 x g for about 5 minutes and the pellet re-suspended in SMCGM for cell counting using a hemacytometer. After centrifugation, the cell pellet was re-suspended in SMCGM at a final concentration of about 2.5 - 6.0 x 10^6 cells per 6-8 ml and transferred into a syringe in preparation for graft seeding. Grafts having an internal diameter of 2.5 mm were seeded with about 2.5-3.5 x 10^6 cells/7 cm graft in about 6 ml SMCGM and 4.0 mm internal diameter grafts were seeded with about 4.0 - 6.0 x 10^6 cells/7 cm graft in about 8 ml SMCGM.

Smooth muscle cell seeding of a graft was performed by attaching the SMC-containing syringe to the proximal connector of the wetted graft and gently forcing the cell suspension into the graft and the media through the base material graft wall. The proximal fitting was then plugged and the smooth muscle cell seeded graft placed into a 16 mm culture tube filled with SMCGM with the graft wedged in the culture tube to prevent it from rolling in the tube. The culture tubes were capped securely and placed into an incubator at about 37°C on a roller apparatus turning at about 10-50 rev/hr. The medium in the culture tubes was replaced at least twice weekly and grafts were cultured for a minimum of about ten days before further processing or the addition of endothelial cells.

A layer of endothelial cells (12) attached to a substratum of smooth muscle

cells 20 adhered to a synthetic base material (24) is shown in Figure 4. This structure may be created in a variety of ways, with the preferred method being generating a single cell suspension of endothelial cells, filling the graft lumen with the suspension, and allowing the endothelial cells to attach, grow, and spread on the SMC surface to form a substantially confluent endothelial cell layer. Alternatively, small patches of endothelial cells may be directly harvested from a donor vessel and the patches seeded into the graft lumen whereby they attach and proliferate to cover the SMC layer.

One method to isolate endothelial cells (ECs) was by using enzymatic methods to release ECs from arterial or venous vessels obtained from dogs. The vessel lumina were cannulated in a laminar flow hood, rinsed with HBSS, and filled with an endothelial cell harvesting enzyme solution (for example, collagenase, dispase, trypsin, etc.) in CMF-HBSS for about 15 minutes at 37°C. Endothelial cells were flushed into a sterile centrifuge tube and the ECs pelleted at 300 x g for 5 minutes. Cells were then plated onto T-25 tissue culture flasks and grown at 37°C until nearly confluent, then passaged. Endothelial cell type was confirmed by morphological criteria, positive staining for Factor VIII, and uptake of acetylated low density lipoprotein.

For graft-seeding purposes, subconfluent endothelial cells (about passages 2-10) were rinsed briefly with CMF-HBSS and washed in CMF-HBSS for about 5 minutes. Cells were harvested by using trypsin-EDTA to release cells from the flasks followed by trypsin neutralization with complete Endothelial Cell Growth Media (ECGM; 80% Medium 199, 16% fetal bovine serum, 2 mM glutamine, 15 units/ml heparin, 25 μg/ml gentamicin, 12.5 μg/ml Endothelial Cell Growth Supplement (Collaborative Biomedical Products, Bedford, MA)). Cells were pelleted at about 300 x g for about 5 minutes, and the pellet re-suspended in ECGM at a final concentration of 1.1 - 1.3 x 10^6 cells/ml. The cell suspension was transferred into a syringe.

A previously seeded SMC-graft (detailed above) was prepared for

endothelial cell seeding by removing both end plugs and briefly rinsing the graft lumen with HBSS. The syringe containing the endothelial cell suspension was then attached to the proximal connector of the SMC-graft and the graft lumen filled with the cell suspension without forcing fluid through the graft wall. The syringe fittings were plugged and the grafts placed into 16 mm culture tubes filled with ECGM. Grafts were wedged into the tubes so they could not rotate independently of the culture tube.

Once the composite structure shown in Figure 4 of endothelial cells (12) and smooth muscle cells (20) on a synthetic base material (24) is created, it is cultured. The following culture conditions have proven successful. Culture tubes were incubated at about 37°C in a roller apparatus turning at about 10-50 revolutions/hr. The medium in the culture tubes was replaced at least twice weekly with ECGM for about 7 days to establish the ECs and then switched to SMCGM for the remainder of the culture period, a minimum of about 10 days total culture time.

Following culturing, a layer of subendothelial matrix (14) will form between the endothelial cells (12) and the smooth muscle cells (20) in the manner shown in Figure 5. Generally the subendothelial matrix layer is less than about one μm in thickness.

Once a suitable layer of subendothelial matrix (14) is created, the endothelial cell (12) layer is removed, such as through one of the following processes. Preferably, the endothelial cells are removed by rinsing the graft about three times with HBSS, treating the endothelialized surface with a stripping solution, such as, an ammonium hydroxide (NH_4OH) solution at a concentration of about 0.025 M, for about 4-4.5 minutes and rinsed again about three times in HBSS. Other suitable treatments may include NH_4OH at a concentration of about 0.01 to about 0.5 M for about 30 seconds to about 60 minutes. Other possibly effective methods of removing the endothelial cells include air drying, or treatment with other stripping solutions, for example, chloroform, methanol, sodium hydroxide, or sodium chloride, either alone or in combination. Other treatments

known to those skilled in the art may also be suitable. Once the cell layer is removed, the structure resembles that shown in Figure 6. Figure 7 shows the structure of the tubular graft form 26 of one embodiment of the present invention.

Scanning electron microscopy inspection of representative samples treated in the above manner confirmed near total loss of the native endothelium and the maintenance of the subendothelial matrix layer. It is understood that for purposes of the present invention, removal of native endothelium from the subendothelial matrix layer in amounts greater than about 80% is considered to render the subendothelial matrix layer substantially free of donor endothelial cells.

The subendothelial matrix analogue exposed after removal of the endothelial cells contains numerous extracellular matrix components. Using immunocytochemical assays, it has been determined that the matrix contains chondroitin sulfate proteoglycans, collagen I, collagen III, collagen IV, elastin, and fibronectin all of which are present both on the exposed blood contact surface as well as within portions of the graft wall. In addition, laminin is an abundant component of the subendothelial matrix blood contact surface and is present to a lesser degree within the graft wall.

Following EC removal, the grafts may be treated with a fixative to preserve the subendothelial matrix layer, to reduce immunogenicity, and to sterilize the graft. This fixation is accomplished by placing the graft into a fixing solution, such as, glutaraldehyde at a concentration of about 0.1-2.5%, for example, in a suitable buffer for about 1-72 hours depending upon the concentration of glutaraldehyde used. Suitable buffers may include N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), acetate, 2-(N-morpholino) ethanesulfonic acid (MES), 3-[N-morpholino] propanesulfonic acid (MOPS), tris hydroxymethyl aminomethane, phosphate, and others. Other fixatives may also be suitable, such as, formaldehyde, dialdehyde starches, ethanol, and polyepoxy compounds, for example. Alternatively, the subendothelial matrix may be used without fixation.

In the preferred embodiment, the subendothelial matrix layer of the graft is

fixed in greater than about 0.5% glutaraldehyde in 20 mM HEPES buffer for a minimum of about two hours. The fixed grafts are rinsed three times in sterile normal saline and washed for a minimum of about 24 hours in fresh sterile saline and then stored at about 4°C. Other suitable fixatives, such as formaldehyde, may be used in addition to glutaraldehyde to assure sterility, for example.

In a preferred embodiment, a bioactive species is attached to the preserved subendothelial matrix layer through chemically reactive functional groups of the components of the subendothelial matrix layer. For example, the proteinaceous components of the subendothelial matrix layer contain unreacted hydroxyl groups, amine groups, carboxyl groups, and thiol groups, among others, to which bioactive species can be attached. In many cases, unreacted chemically functional groups of the polysaccharide moieties of many of the subendothelial matrix proteins can be utilized to attach bioactive species to the subendothelial matrix layer.

In addition, unreacted chemically functional groups of the agent used to preserve the subendothelial matrix may be utilized to attach bioactive species to the subendothelial matrix layer. For example, when an aldehyde-containing preservative, such as glutaraldehyde, is used to cross-link proteinaceous components of the subendothelial matrix layer together, any unreacted aldehyde groups of the cross-linked subendothelial matrix layer can be readily utilized to attach bioactive species to the matrix.

Alternatively, a bioactive species can be attached to a non-preserved subendothelial matrix layer using available unreacted chemically functional groups of the components of the subendothelial matrix layer (See Figure 6A, for example).

Regardless of which chemically functional group of a subendothelial matrix is utilized as an attachment point for a bioactive species, mild conjugation schemes are preferred for attachment of the bioactive species to the matrix in order to maintain, or maximize, the bioactivity of the attached bioactive species and the matrix.

Optionally, an intermediate, or spacer, compound is placed in between the bioactive species and the subendothelial matrix layer in attaching the bioactive species to the matrix (See Figure 6B, for example). Suitable compounds for use as a spacer compound in the present invention include, but are not limited to, ethylene diamine, putrescine, succinic acid, diaminohexane, polyethylene glycol diamine, polyethyleneimine, glyoxylic acid, short chain polyethylene glycol, and glycine, for example.

The covalent attachment of bioactive species to a subendothelial matrix layer is generally non-reversible, i.e., the bioactive species is not readily released from the subendothelial matrix layer. Spacers capable of selectively releasing an attached bioactive species have utility in controlled delivery of the bioactive species to an implant recipient. Selective release of a bioactive species from a subendothelial matrix layer is performed by cleaving the spacer compound under appropriate reaction conditions including, but not limited to, photon irradiation, enzymatic cleavage, oxidation/reduction reactions, or hydrolysis, for example. The selective cleavage and release of attached bioactive species may be accomplished using techniques such as those described by Horton et al. in "Covalent Immobilization Of Proteins By Techniques Which Permit Subsequent Release," Meth. Enzymology, 135: 130 (1987); S. Wong, "Chemistry Of Protein Conjugation and Cross-Linking, CRC Press (1991); and U.S. Patent No. 4,745,160, issued to Churchill et al., which is incorporated herein by reference. Suitable compounds for use as cleavable tethers, or spacer compounds, include, but are not limited to, polyhydroxyacids, polyanhydrides, polyamino acids, tartarates, and cysteine-linkers, such as Lomant's Reagent, for example.

Bioactive species are attached to a preserved subendothelial matrix layer by covalent reaction with unreacted moieties of the fixative compound, or by bioconjugation via reactive moieties present on the preserved subendothelial matrix introduced through chemical activation schemes. In the first scenario, fixative is added to the subendothelial matrix to chemically cross-link its

constituent proteins to effect preservation. However, when the fixative is added in excess, unreacted free aldehyde groups will be present after fixation is completed; these unreacted free aldehyde groups may then be reacted with bioactive species to attach the species onto the subendothelial matrix. For example, after fixation of the subendothelial matrix with glutaraldehyde and subsequent rinsing in saline, the graft is then immersed in a buffered solution (pH 6 - 9) containing the bioactive species, at a concentration of about 0.01 - 10%, for a period of about 1 to 72 hours. Following attachment of the bioactive species onto the aldehyde free end groups, the construction is rinsed in saline. The construction may then by treated with a solution, such as about 0.01-1 M sodium cyanoborohydride in a buffered solution at pH 6-10 for about 1-24 hours, to stabilize the imine bond thus formed, thereby stabilizing the bioactive species on the matrix surface.

Alternatively, the bioactive species may be attached directly onto the proteinaceous components of the preserved subendothelial matrix, rather than relying upon the presence of unreacted free aldehyde end groups from the fixative. Constituent proteins and proteoglycans of the preserved matrix can be activated using suitable activation protocols; the activated matrix constituents may then be reacted with bioactive species to graft the bioactive species directly onto the matrix. For example, after fixation in glutaraldehyde and subsequent rinsing in saline, the construct is then immersed in a buffered solution of about 1 - 10% 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC), about pH 5 - 10, for about 0.5 - 6 hr at about 4 - 25°C. The activated construct is then rinsed in saline, and then immersed in a buffered solution of bioactive species at a concentration of about 0.01 - 10%, for about 1-24 hours at about 4 - 25°C. Following attachment of the bioactive species onto the activated subendothelial matrix, the graft is rinsed in saline. Optionally, the construct may be preserved at this stage by treatment with a fixative, such as 0.5-5.0% glutaraldehyde, for example. Alternatively, the EDC and the bioactive species may both be present, to allow activation of and attachment to the subendothelial matrix to proceed simultaneously. Activation protocols

relationships. After extended co-culture, the subendothelial matrix is produced between the cell layers. The subendothelial matrix layer may then be exposed as outlined above and processed accordingly.

While VSMCs are the preferred use as the substrate cell layer in the production of the subendothelial matrix layer of the present invention, it is recognized that it may be possible to use other cell types to provide a similar function. Among the other potential cell types are smooth muscle cells from the digestive system or urinary tract, as well as fibroblasts, among others. It may also be possible to produce a suitable subendothelial matrix layer for use with the present invention through the use of other, non-living, base materials against which a subendothelial matrix layer may be formed by an attached endothelial cell layer.

Additionally, while vascular endothelial cells are the preferred embodiment, it may also be possible to use other endothelial cell types to produce the subendothelial matrix layer in conjunction with the VSMCs, SMCs, fibroblasts, or other similar cell types. These cell types may include, for example, microvascular endothelial cells, corneal endothelium, glomerular epithelium, and mesothelial cells, among others.

It will be recognized that the present invention may be produced in different physical configurations depending upon the specific blood interface application. As shown in the structure of Figure 8, it is possible to create the subendothelial matrix 14 on an SMC layer (20) on a synthetic base material (24) in a flat sheet form (28). Another application of the present invention shown in Figure 9 includes the use of the subendothelial matrix layer (14) on the surfaces of a heart valve (30).

Without intending to limit the scope of the present invention, the following examples illustrate how the present invention can be made and used.

EXAMPLES

EXAMPLE 1

The following is an example of grafts of the present invention (inventive grafts) compared to ePTFE grafts (control grafts) of the same diameter in implant studies. The inventive grafts were fabricated as described in the detailed description above. Briefly, endothelial cells (ECs) were seeded onto a vascular smooth muscle cell-covered (VSMC-covered) 2.5 mm ePTFE tube and cultured together for a minimum of 10 days. The inventive grafts were then treated for 30 seconds with 0.25 M NH_4OH to remove the endothelial cells and rinsed by flushing with HBSS. After rinsing, the inventive grafts were fixed with 2.5% glutaraldehyde in 0.2 M Sorenson's phosphate buffer (pH 7.0) for 2 hours. Following fixation, the grafts were rinsed in two changes of sterile normal saline, and stored at 4°C in sterile normal saline until required for implantation.

The inventive and control grafts were implanted into greyhound dog brachial arteries as test pairs using a control graft consisting of a 2.5 mm diameter, 30 μm ePTFE tubing. One brachial artery received the inventive graft and the contralateral brachial artery received the 30 μm ePTFE control graft. The grafts, 2.5 cm in length, were implanted using standard end-to-end surgical technique. No anticoagulants or antiplatelet agents were administered at any time. Each dog was followed daily using a doppler ultrasound duplex scanner for the first two weeks postoperatively and weekly thereafter. Contrast angiography was also employed to determine patency status in the event of ambiguous ultrasound results. At one month, it was found that the inventive grafts were patent in three of six (3/6) cases and the ePTFE control grafts were patent in zero of six (0/6) cases. In conclusion, the inventive graft showed an improved patency performance compared to control ePTFE grafts of the same diameter.

EXAMPLE 2

This is an example of an alternative method of fabricating the graft of the present invention described in Example 1 above. Individual cultures of vascular SMCs and ECs were harvested for graft fabrication by removing growth medium, rinsing the cells with CMF-HBSS, and washing the cells in CMF-HBSS for about 3-5 minutes. The CMF-HBSS was then removed and 1.5-3.0 ml of trypsin-EDTA (depending on flask size) was added to release cells from the flask. Complete SMCGM was added to the individual flasks to inactivate the trypsin, and the cells were triturated and pelleted by centrifugation at 300 x g for 5 minutes. The supernatant was discarded, the cells were re-suspended in SMCGM, cell counts were carried out, and a second pelleting of cells was performed. The final individual SMC and EC cell pellets were re-suspended in SMCGM at a concentration of about 1 x 10^6 cells/ml. The cell types were mixed in EC:SMC ratios of 1:9, 1:4, and 1:2.

Synthetic base materials consisting of porous ePTFE tubes of 2.5 mm internal diameter and 30 μm fibril length were cut to 7 cm and mounted onto syringe connector fittings. A polypropylene plug was fitted onto the distal ends of the tube. The PTFE was wetted with 100% ethanol. The alcohol was then displaced with HBSS solution by pressurizing the tube, thereby forcing the HBSS through the porous tube wall. The wetted tubes were then stored in HBSS until seeding with the vascular cells.

The inventive graft was seeded with smooth muscle cells and endothelial cells, using positive pressure, by placing 2.5-3.5 x 10^6 cells of the mixed SMC-EC suspensions into a total volume of about 6 ml SMCGM in a syringe. The 2.5 mm diameter size inventive graft was seeded with 2.5-3.5 x 10^6 cells/7 cm graft. Cell numbers were quantified using a hemacytometer. The syringe containing the cell suspension was attached to the proximal syringe connector on the graft and the cell mixture was gently injected using positive pressure to force the media through the

graft wall depositing the cells on the graft luminal surface. After placing cells into the graft, a second polypropylene plug was attached to the open connector to seal the cells in the graft luminal space.

The inventive seeded graft was placed into a 16 mm culture tube filled with SMCGM. The grafts were wedged in the culture tubes, capped securely, and placed in a roller apparatus turning at about 10 rev/hr. in a 37°C incubator. Seeded grafts were cultured for 7 to 10 days with fresh medium feedings every 2-4 days. During this culture period, the endothelial cells were segregated from the SMCs forming a confluent EC monolayer on top of the SMCs. Scanning electron microscopy analysis and staining with acetylated low density lipoprotein were used to verify the presence of ECs on the graft luminal surface.

The inventive grafts were then rinsed three times with HBSS, and the lumen treated with 0.025 or 0.25 M NH_4OH for 4.5 and 3.5 minutes, respectively, to remove the ECs. The grafts were rinsed with HBSS, fixed with 0.25% glutaraldehyde for 24 hours at 23°C, followed by extensive washing in sterile normal saline. This resulted in the production of a subendothelial matrix layer similar to that produced in Example 1 described above.

EXAMPLE 3

The following is an example of how the present invention may be practiced employing a substratum layer of fibroblast cells. A 30 μm ePTFE base tube was wetted with 100% ethanol and flushed with HBSS to displace the ethanol from the graft interstices. Dog foreskin fibroblasts were then harvested and applied to the graft surface using positive pressure to force the cells against the lumen of the graft wall. The graft was then placed into culture for a period of about 10-14 days. Following this, endothelial cells were seeded onto the fibroblast matrix surface by filling the graft lumen with a suspension of endothelial cells at a concentration of about 1.1-1.3 x 10^6 cells/ml of ECGM. The graft was wedged into a culture tube

and placed into a 37°C incubator on a roller apparatus for at least 10 days to allow the endothelial cells to establish a monolayer and become firmly attached. Endothelial cell coverage was confirmed by observing uptake of acetylated low density lipoprotein.

Following establishment of the endothelial cell-fibroblast co-culture to provide a subendothelial matrix layer, the endothelial cells were selectively removed by treatment with a stripping solution. After the endothelial cells were removed, the graft was fixed in glutaraldehyde to preserve, sterilize, and stabilize the subendothelial matrix. Following fixation, the graft is washed extensively with a sterile saline solution and stored in same until use.

EXAMPLE 4

In this example, a vascular graft is first fabricated as specified in Example 1 having a preserved subendothelial matrix layer. Following fixation of the subendothelial matrix layer in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), the graft was rinsed in 0.1M carbonate/bicarbonate buffer (pH 9.5). A spacer compound comprised of 1% ethylene diamine in 0.1M carbonate/bicarbonate buffer (pH 9.5) was attached to the fixed, or preserved, subendothelial matrix layer by treating the preserved matrix with the ethylene diamine solution for about 2 hours at about 37°C.

The preserved subendothelial matrix layer having the ethylene diamine spacer compound attached thereto is then briefly rinsed in saline and further treated with 2.5% glutaraldehyde in 0.1M borate buffer, pH 8.5, for about 16 hours at about 23°C. Following this step, the graft was incubated with the bioactive species fibronectin at a concentration of about 100μg/ml carbonate buffer (pH 9.5) for about 2 hours at about 37°C. The graft is then rinsed in normal saline and stored at about 4°C until use.

EXAMPLE 5

In this example, a subendothelial matrix layer is initially fabricated in accordance with Example 1. Following a treatment with NH_4OH to remove endothelial cells from the subendothelial matrix layer, the matrix material is treated with 2% 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC), pH 7.4, for about 16 hours at about 23°C to activate particular constituents of the subendothelial matrix layer. The graft is then briefly rinsed in normal saline. Following this step, the graft is incubated with the bioactive species fibronectin at a concentration of about 100µg/ml carbonate buffer (pH 9.5) for about 2 hours at about 37°C. The graft is optionally preserved by treating the construction with 2.5% glutaraldehyde in phosphate buffered saline for about 2 hours at about 23°C. The resultant graft is expected to provide enhanced patency performance under *in vivo* conditions.

EXAMPLE 6

This example illustrates reversible attachment of a bioactive species to a subendothelial matrix layer prepared in accordance with Example 1. In this example, a vascular graft is constructed according to Example 1. Following preservation of the subendothelial matrix layer with about 2.5% glutaraldehyde, the graft is reacted with about 0.5M sodium cyanoborohydride ($NaCNBH_3$) in a carbonate/bicarbonate buffer (pH 9.5) for about 3 hours at room temperature. The graft is extensively washed in carbonate/bicarbonate buffer. After the washing step, the graft is exposed to 1-amino 3-propanol in a carbonate/bicarbonate buffer (pH 9.5) for about 3 hours at room temperature. The graft is again extensively washed in carbonate/bicarbonate buffer.

A bioactive species in the form of the drug methotrexate is bound through an ester bond to the preserved subendothelial matrix. This is accomplished by placing the graft in a 0.25M solution of methotrexate in dimethyl formamide

(DMF) at room temperature and then cooled to about 0°C and reacted for about 2 hours. The graft containing solution is then allowed to return to room temperature. The reaction is allowed to continue for an additional 2 hours at room temperature with stirring. The graft is then washed extensively in DMF, ethanol, and water, respectively. The resultant graft having methotrexate attached to the preserved subendothelial matrix layer is stored lyophilized and stored under refrigeration until use. The attachment of methotrexate to the preserved subendothelial matrix layer is reversible. To release the methotrexate from the subendothelial matrix layer, the ester bond attaching the drug to the matrix is cleaved by hydrolysis.

EXAMPLE 7

This example illustrates a non-reversible attachment of a bioactive species to a subendothelial matrix layer prepared in accordance with Example 1. In this example, a vascular graft is constructed according to Example 1. Following preservation of the subendothelial matrix layer with about 2.5% glutaraldehyde, the graft is reacted with about 0.5M sodium cyanoborohydride ($NaCNBH_3$) in a carbonate/bicarbonate buffer (pH 9.5) for about 3 hours at room temperature. The graft is extensively washed in carbonate/bicarbonate buffer. After the washing step, the graft is exposed to a bioactive species in the form of a drug containing at least one reactive amino group, such as meso-chlorin e_6 monoethylene diamine disodium salt. This drug is attached to pendant aldehyde groups of the preserved subendothelial matrix layer.

To attach meso-chlorin e_6 monoethylene diamine disodium salt to the subendothelial matrix layer, a solution of the drug at a concentration of about 0.25M in a carbonate/bicarbonate buffer (pH 9.5) is exposed to the graft overnight at room temperature in the dark. The graft is then extensively washed in carbonate/bicarbonate buffer (pH 9.5). The washed graft is exposed to about 0.5M sodium cyanoborohydride in a carbonate/bicarbonate buffer (pH 9.5) for about 3

hours. Following this reaction, the resultant graft is extensively washed in saline and stored in same at about 4°C until use.

EXAMPLE 8

This example illustrates the attachment of bioactive species in the form of a peptide to a preserved subendothelial matrix layer. In this example, a vascular graft is constructed according to Example 1. Following preservation of the subendothelial matrix layer with about 2.5% glutaraldehyde, the graft is placed in a carbonate/bicarbonate buffer (pH 9.5) and a peptide having the sequence glycine-arginine-glycine- asparagine-serine-proline, which contains the cell anchorage amino acid sequence arginine-glycine-asparagine (arg-gly-asp) is added to the buffer. The reaction is permitted to proceed overnight at about 37°C. The reaction solution is removed and fresh carbonate/bicarbonate buffer (pH 9.5) is added. 20 mg of sodium borohydride is added to the carbonate/bicarbonate buffer. The reaction is allowed to proceed overnight at room temperature. The resultant graft is washed extensively in normal saline, ethanol, and normal saline, respectively. The prepared graft is stored in saline until seeded with endothelial cells.

Endothelial cells at a concentration of about 150,000 cells/ml are added to the prepared graft in phosphate buffered saline (pH 7.4) to allow attachment of the cells to the arg-gly-asp containing peptide bound to the subendothelial matrix layer. The cells are allowed to proliferate for several days.

The presence of endothelial cells attached to the subendothelial matrix layer is confirmed with 9-6-diamine-Z-phenyl-indole dihydrochloride.

EXAMPLE 9

This example illustrates the immobilization of lysine to a preserved subendothelial matrix layer such that the lysine molecules are immobilized

through the α-amine group of the molecule, while leaving the ε-amine group of the lysine molecules are unreacted. This method relies on Boc protection of the ε-amine group of the lysine molecules.

A construction of the present invention having a preserved subendothelial matrix layer is immersed in a 5% solution of carbonyldiimidazole dissolved in anhydrous acetone for 60 minutes. The construction is successively rinsed in acetone, then aqueous buffer (150mM $NaHCO_3$, 500mM NaCl, pH 8.0, 4°C). N-ε-Boc-L-lysine (Bachem California, Torrance CA) is dissolved in the coupling buffer at a concentration of 10mg/ml. The construct is immersed in this solution for 24 hr. at 4°C with gentle agitation.

The construction is rinsed successively in coupling buffer, deionized water, and tetrahydrofuran. The construction is then immersed in a solution of 10% trifluoroacetic acid dissolved in tetrahydrofuran for 60 min. This is followed by successive rinsing in tetrahydrofuran, deionized water, and coupling buffer. The resulting article contains lysine immobilized to the preserved subendothelial matrix layer exclusively via the α-amine terminus with the ε-amine group of the lysine unreacted, or free.

EXAMPLE 10

This example illustrates another immobilization of lysine to a preserved subendothelial matrix layer such that the lysine molecules are immobilized through the α-amine group of the molecule, while leaving the ε-amine group of the lysine molecules are unreacted. This method relies on Boc protection of the ε-amine group of the lysine molecules.

A construction of the present invention having a preserved subendothelial matrix layer is immersed in a 5% solution of carbonyldiimidazole dissolved in anhydrous dimethylformamide for 60 minutes. The construction is rinsed in

dimethylformamide. N-ε-Boc-L-lysine (Bachem California, Torrance CA) is dissolved in dimethylformamide at a concentration of 10mg/ml. The construction is immersed in this solution for 24 hr at 4°C with gentle agitation. The construction is then rinsed in dimethylformamide. Next, the sample is immersed in a solution of 10% trifluoroacetic acid dissolved in dimethylformamide for 60 min., followed by successive rinsing in dimethylformamide, deionized water, and phosphate buffered saline. The resulting article contains lysine immobilized to the preserved subendothelial matrix layer exclusively via the α-amine terminus with the ε-amine group of the lysine unreacted, or free.

EXAMPLE 11

This example illustrates another immobilization of lysine to a preserved subendothelial matrix layer such that the lysine molecules are immobilized through the α-amine group of the molecule, while leaving the ε-amine group of the lysine molecules are unreacted. This method relies on FMOC protection of the ε-amine group of the lysine molecules.

A construction of the present invention having a preserved subendothelial matrix layer is immersed in a 5% solution of carbonyldiimidazole dissolved in anhydrous acetone for 60 minutes. The construction is successively rinsed in acetone, then aqueous buffer (150mM $NaHCO_3$, 500mM NaCl, pH 8.0, 4°C). N-ε-FMOC-L-lysine (Bachem California, Torrance CA) is dissolved in the coupling buffer at a concentration of 10mg/ml. The construct is immersed in this solution for 24 hr. at 4°C with gentle agitation.

The construction is rinsed successively in coupling buffer, deionized water, and tetrahydrofuran. The construction is then immersed in a solution of 10% piperidine dissolved in tetrahydrofuran for 60 min. This is followed by successive rinsing in tetrahydrofuran, deionized water, and coupling buffer. The resulting

article contains lysine immobilized to the preserved subendothelial matrix layer exclusively via the α-amine terminus with the ε-amine group of the lysine unreacted, or free.

EXAMPLE 12

This example illustrates another immobilization of lysine to a preserved subendothelial matrix layer such that the lysine molecules are immobilized through the α-amine group of the molecule, while leaving the ε-amine group of the lysine molecules are unreacted. This method relies on FMOC protection of the ε-amine group of the lysine molecules.

A construction of the present invention having a preserved subendothelial matrix layer is immersed in a 5% solution of carbonyldiimidazole dissolved in anhydrous dimethylformamide for 60 minutes. The construction is rinsed in dimethylformamide. N-ε-FMOC-L-lysine (Bachem California, Torrance CA) is dissolved in dimethylformamide at a concentration of 10mg/ml. The construction is immersed in this solution for 24 hr at 4°C with gentle agitation. The construction is then rinsed in dimethylformamide. Next, the sample is immersed in a solution of 10% trifluoroacetic acid dissolved in dimethylformamide for 60 min., followed by successive rinsing in dimethylformamide, deionized water, and phosphate buffered saline. The resulting article contains lysine immobilized to the preserved subendothelial matrix layer exclusively via the α-amine terminus with the ε-amine group of the lysine unreacted, or free.

While particular embodiments of the present invention have been illustrated and described herein, the present invention should not be limited to such illustrations and descriptions. It should be apparent that changes and modifications may be incorporated and embodied as part of the present invention

within the scope of the following claims.

CLAIMS

1. A blood contact material comprising:

a synthetic base material;

a first layer of cells attached to the synthetic base material;

a second layer attached to the first layer wherein the second layer comprises subendothelial matrix substantially free of endothelial cells;

wherein the subendothelial matrix layer serves as a direct blood contact surface; and

a bioactive species attached to the subendothelial matrix layer.

2. The blood contact material of claim 1 wherein the first layer is comprised of fibroblasts.

3. The blood contact material of claim 1 wherein the first layer is comprised of smooth muscle cells.

4. The blood contact material of claim 3 wherein the smooth muscles cells are vascular smooth muscle cells.

5. The blood contact material of claim 1 wherein the second layer is preserved.

6. The blood contact material of claim 1 wherein the material is formed through the following process:

culturing a first layer of smooth muscle cells on the synthetic base material;

culturing on the first layer of smooth muscle cells a layer of endothelial cells, the interaction of the smooth muscle cells and the endothelial cells forming a subendothelial matrix layer;

stripping the endothelial cell layer from the subendothelial matrix layer to produce the second layer comprised of subendothelial matrix layer substantially free of endothelial cells;

attaching a bioactive species to the subendothelial matrix layer; and

employing the second layer and attached bioactive species as a direct blood contact surface.

7. The process of claim 6 which further comprises:

removing the second layer from the smooth muscle cells; and

applying the second layer to a separate synthetic base material or recipient vascular tissue for use as a direct blood contact surface.

8. The process of claim 6 wherein the synthetic base material is selected from the group consisting of at least one of polytetrafluoroethylene, polyethylene terepthalate, fluorinated ethylene propylene, polyethylene, polypropylene, and siloxane.

9. The process of claim 6 which further comprises:

stripping the endothelial cell layer from the subendothelial matrix layer by treating the endothelial cell layer with a stripping solution.

10. The process of claim 9 wherein the stripping solution is selected from at least one of a group consisting of ammonium hydroxide, chloroform, methanol, and sodium chloride.

11. The blood contact material of claim 1 wherein the material is formed through the following process:

culturing a first layer of smooth muscle cells on the synthetic base material;

culturing on the first layer of smooth muscle cells a layer of endothelial cells, the interaction of the smooth muscle cells and the endothelial cells forming a subendothelial matrix layer;

stripping the endothelial cell layer from the subendothelial matrix layer to produce a second layer comprised of subendothelial matrix layer substantially free of endothelial cells;

preserving the second layer;

attaching a bioactive species to the second layer; and

employing the preserved second layer and attached bioactive species

as a direct blood contact surface.

12. The process of claim 11 which further comprises:

removing the second layer from the smooth muscle cells; and

applying the second layer to a separate synthetic base material or recipient vascular tissue for use as a direct blood contact surface.

13. The process of claim 11 wherein the synthetic base material is selected from the group consisting of at least one of polytetrafluoroethylene, polyethylene terepthalate, fluorinated ethylene propylene, polyethylene, polypropylene, and siloxane.

14. The process of claim 11 which further comprises:

stripping the endothelial cell layer from the subendothelial matrix layer by treating the endothelial cell layer with a stripping solution.

15. The process of claim 14 wherein the stripping solution is selected from at least one of a group consisting of ammonium hydroxide, chloroform, methanol, and sodium chloride.

16. The blood contact material of claim 1 wherein

the synthetic base material comprises a polymeric material to which the first layer will adhere.

17. The blood contact material of claim 16 wherein

the synthetic base material is selected from the group consisting of at least one of polytetrafluoroethylene, polyethylene terepthalate, fluorinated ethylene propylene, polyethylene, polypropylene, and siloxane.

18. The blood contact material of claim 1 wherein

the synthetic base material comprises a tube having an exterior and an interior;

wherein the first layer is attached to the interior of the tube; and

wherein the second layer is attached to the first layer to form a covering.

19. The blood contact material of claim 20 wherein

the tube having the subendothelial matrix layer covering serves as an implantable appliance.

20. The blood contact material of claim 18 wherein

the synthetic base material is selected from the group consisting of at least one of polytetrafluoroethylene, polyethylene terepthalate, fluorinated ethylene propylene, polyethylene, polypropylene, and siloxane.

22. The blood contact material of claim 5 wherein

the synthetic base material comprises a tube having an exterior and an interior;

wherein the first layer is attached to the interior of the tube; and

wherein the preserved second layer is attached to the first layer to form a covering.

23. The blood contact material of claim 22 wherein

the tube having the preserved subendothelial matrix layer covering serves as an implantable appliance.

24. The blood contact material of claim 1 wherein

the second layer contains at least one protein in a group consisting of chondroitin sulfate proteoglycans, collagen I, collagen III, collagen IV, elastin, laminin, and fibronectin.

25. The blood contact material of claim 5 wherein

the preserved second layer contains at least one protein in a group consisting of chondroitin sulfate proteoglycans, collagen I, collagen III, collagen IV, elastin, laminin, and fibronectin.

26. A method for producing a direct blood contact material which comprises:

providing a layer of smooth muscle cells;

culturing on the layer of smooth muscle cells a layer of endothelial cells;

allowing a subendothelial matrix layer to form between the smooth

muscle cells and the endothelial cells;

stripping away the endothelial cell layer to expose the subendothelial matrix layer;

attaching a bioactive species to the subendothelial matrix layer; and

employing the subendothelial matrix and attached bioactive species as the direct blood contact surface.

27. The method of claim 26 which further comprises:

forming the subendothelial matrix layer into a tubular appliance, the subendothelial matrix layer comprising an interior conduit for the appliance through which blood will flow.

28. The method of claim 26 which further comprises:

stripping away the endothelial cell layer by treating with a stripping solution.

29. The method of claim 28 wherein the stripping solution comprises an aqueous stripping solution of ammonium hydroxide at a concentration ranging from about 0.01M to about 0.5M.

30. The method of claim 26 that further comprises:

stripping away the endothelial cell layer by freeze-thaw treating the endothelial cell layer.

31. The method of claim 26 which further comprises:

stripping the endothelial cell layer by air dry treating the endothelial cell layer.

32. The method of claim 26 which further comprises:

removing the subendothelial matrix layer from the smooth muscle cell layer; and

applying the subendothelial matrix layer to an appliance to serve as a direct blood contact surface thereon.

33. The method of claim 26 which further comprises:

preserving the exposed subendothelial matrix layer; and

employing the preserved subendothelial matrix layer and attached bioactive species as a direct blood contact surface.

34. The method of claim 26 which further comprises:

treating the subendothelial matrix layer with a fixative solution to preserve the subendothelial matrix layer.

35. The method of claim 33 which prior to preserving the exposed subendothelial matrix layer further comprises:

removing the subendothelial matrix layer from the smooth muscle cell layer; and

applying the subendothelial matrix layer and attached bioactive species to an appliance to serve as a direct blood contact surface thereon.

36. A method of making an implantable material having at least one blood contact surface comprising:

procuring vascular smooth muscle cells from a blood vessel;

placing the vascular smooth muscle cells onto a surface of a synthetic base material to form a coating of vascular smooth muscle cells;

applying endothelial cells to the coating of vascular smooth muscle cells to form a layer of endothelial cells on the vascular smooth muscle cells;

culturing a subendothelial matrix layer between the endothelial cell layer and the vascular smooth muscle cell coating;

removing the endothelial cell layer to expose the subendothelial matrix layer, the subendothelial matrix layer being suitable for use as a direct blood contact surface; and

attaching a bioactive species to the subendothelial matrix layer.

37. The method of claim 36 which further comprises:

implanting the synthetic base material having the coating of vascular smooth muscle cells and a subendothelial matrix layer applied thereto, so as to position the subendothelial matrix layer as a direct blood contact surface.

38. The method of claim 36 which further comprises:

removing the subendothelial matrix layer and applying the subendothelial matrix layer to the surface of another synthetic base material.

39. The method of claim 36 which further comprises:

providing synthetic base material in a form selected from a group consisting of a tubular form, the form of a sheet, a spherical form, and the form of a hollow container.

40. The method of claim 39 which further comprises:

providing the synthetic base material in the form of a porous polytetrafluoroethylene.

41. The method of claim 42 which further comprises:

preserving the subendothelial matrix layer prior to attachment of a bioactive species to the subendothelial matrix layer, the preserved subendothelial matrix layer and attached bioactive species being suitable for use as a direct blood contact surface.

42. The method of claim 41 which further comprises:

implanting the synthetic base material having the coating of vascular smooth muscle cells and a preserved subendothelial matrix layer applied thereto, so as to position the preserved subendothelial matrix layer and attached bioactive species as a direct blood contact surface.

43. The method of claim 41 which further comprises:

removing the subendothelial matrix layer and applying the subendothelial matrix layer to the surface of another synthetic base material, prior to preserving the subendothelial matrix layer and attaching bioactive species thereto.

44. A direct blood contact material which comprises:

a subendothelial matrix layer containing at least one protein in a group consisting of chondroitin sulfate proteoglycans, collagen I, collagen III, collagen IV, elastin, laminin, and fibronectin;

a synthetic base material to which the subendothelial matrix layer is

attached;

wherein the subendothelial matrix serves as a direct blood contact surface; and

a bioactive species attached to the subendothelial matrix layer.

45. The blood contact material of claim 44 wherein the synthetic base material is selected from the group consisting of at least one of polytetrafluoroethylene, polyethylene terepthalate, fluorinated ethylene propylene, polyethylene, polypropylene and siloxane.

46. The blood contact material of claim 44 wherein the synthetic base material comprises a porous polytetrafluoroethylene.

47. The blood contact material of claim 44 wherein the synthetic base material comprises a tubular structure, and wherein the subendothelial matrix layer comprises an inside surface of the tubular structure, serving as a direct blood contact surface for a blood conduit.

48. The blood contact material of claim 44 wherein the synthetic base material comprises at least a portion of a heart valve, and wherein the subendothelial matrix layer comprises a direct blood contact surface for the heart valve.

49. The blood contact material of claim 44 wherein the synthetic base material comprises a flexible sheet, and wherein the subendothelial matrix layer comprises a surface on the sheet serving as a direct blood contact surface for the sheet.

50. The blood contact material of claim 1 wherein the bioactive species is lysine.

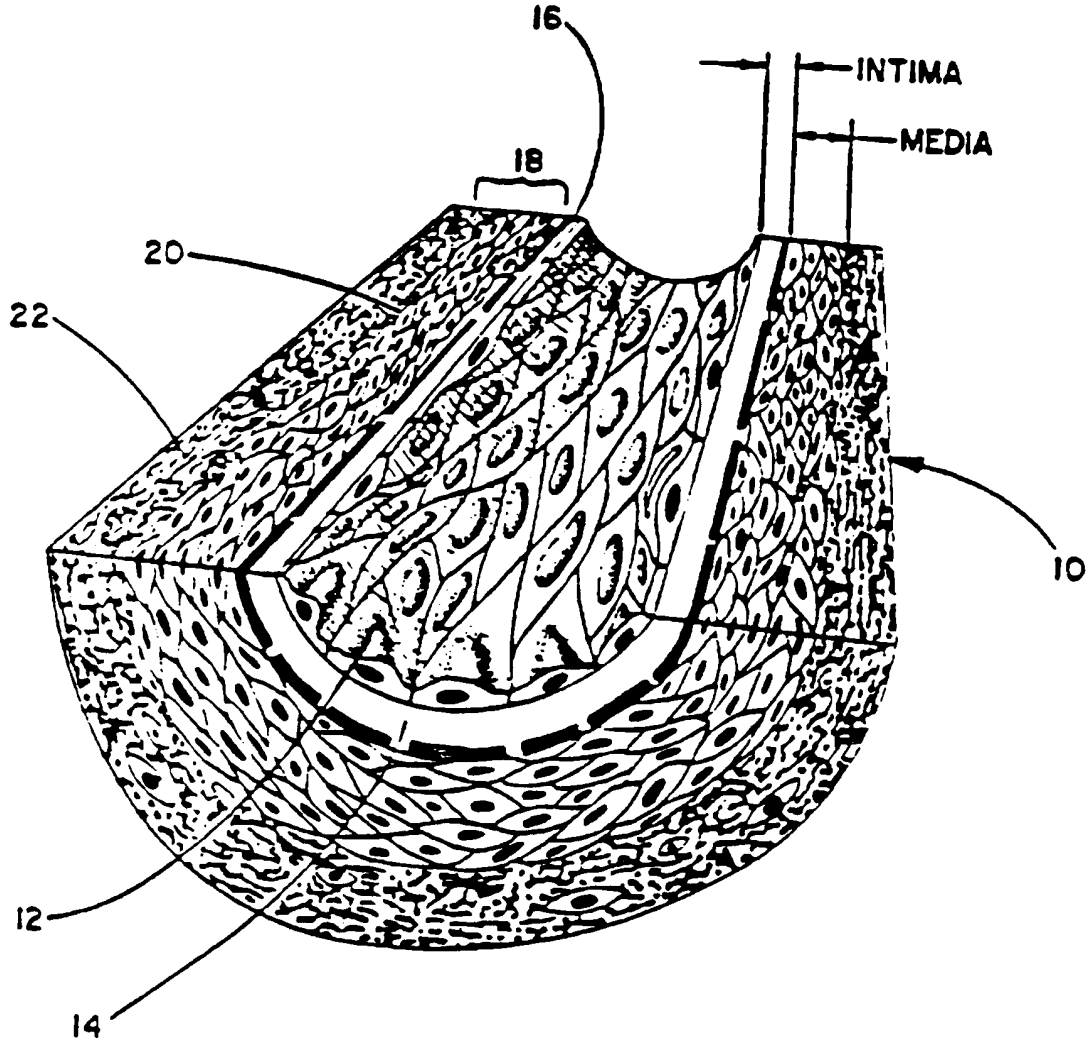

FIG. 1

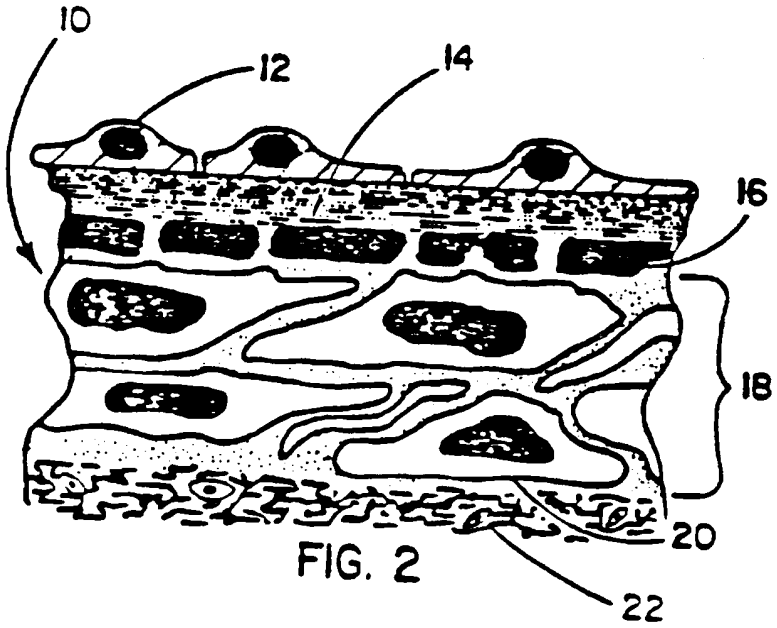

FIG. 2

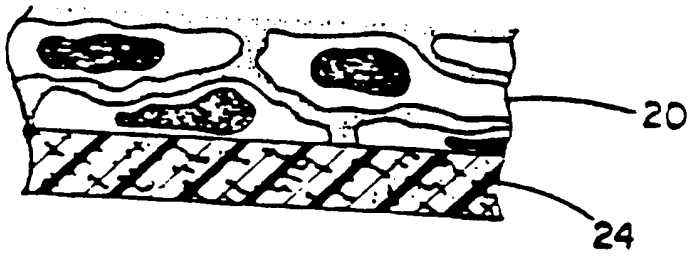

FIG. 3

FIG. 4

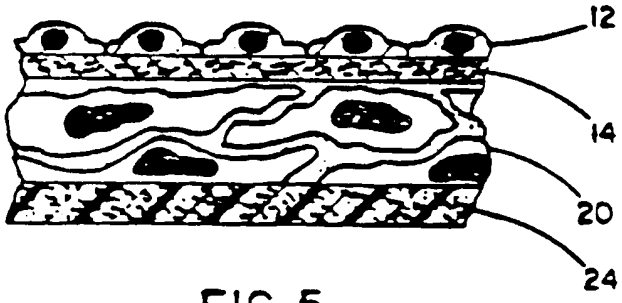

FIG. 5

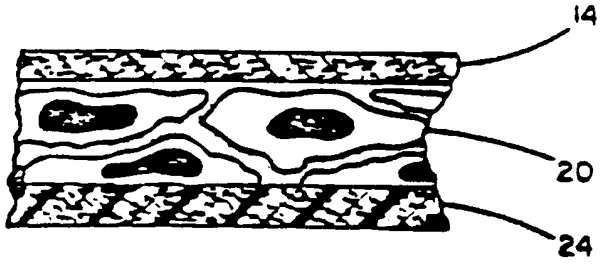

FIG. 6

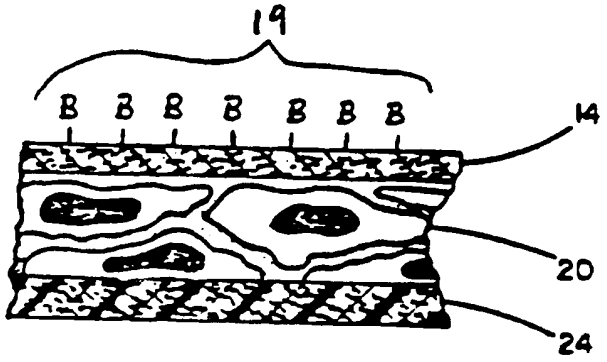

FIG. 6A

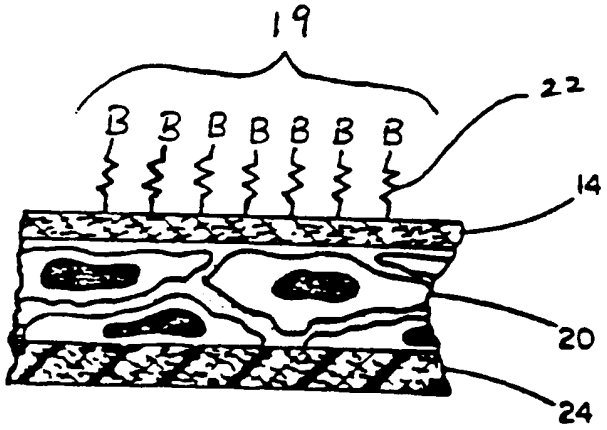

FIG. 6B

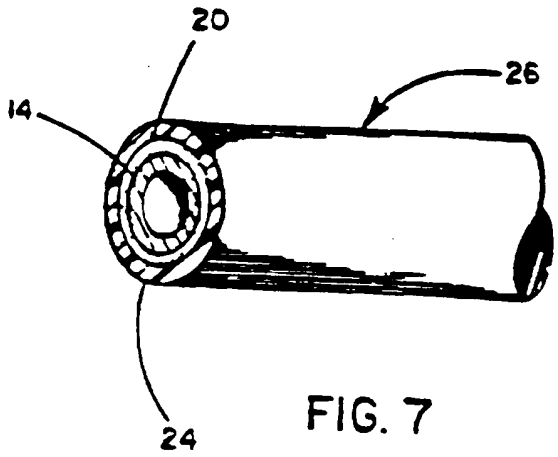

FIG. 7

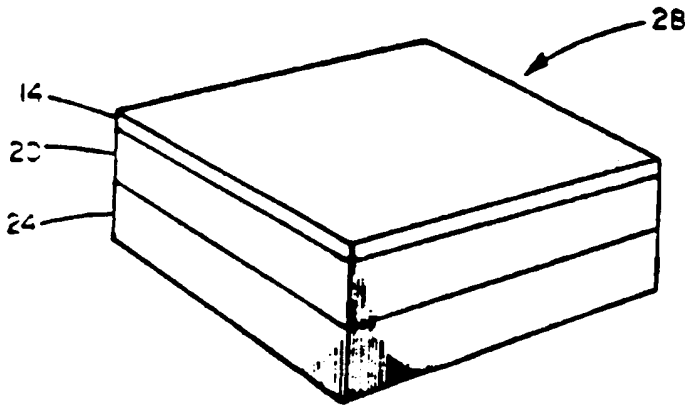

FIG. 8

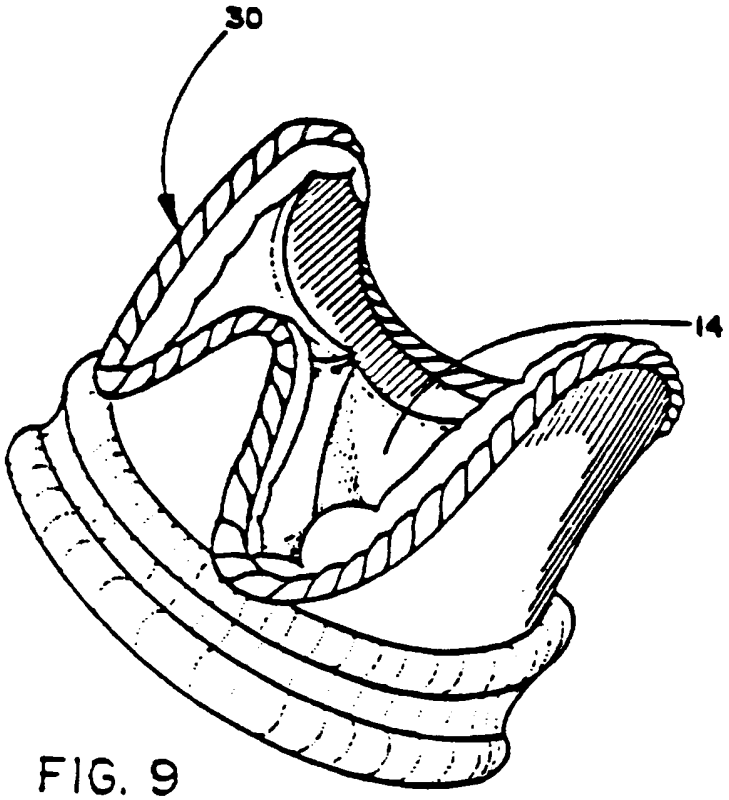

FIG. 9

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 97/09633

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 A61F2/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29714 A (GORE & ASS) 9 November 1995 see claims; figures	1-50
X	WO 95 29713 A (GORE & ASS) 9 November 1995 see figures; examples	1-50
X	WO 95 29712 A (GORE & ASS) 9 November 1995 cited in the application see claims; figures	1-50
A	YOON-SHIN LEE ET AL.: "ENDOTHELIAL CELL SEEDING ONTO THE EXTRACELLULAR MATRIX OF FIBROBLASTS FOR THE DEVELOPMENT OF A SMALL DIAMETER POLYURETHANE VESSEL" ASAIO JOURNAL, vol. 39, no. 3, 1993, HAGERSTOWN, MD, US, pages 740-745, XP000412643 see abstract	1-50

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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2

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1997	05. 11. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/09633

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9529714 A	09-11-95	AU 2369695 A	29-11-95
		CA 2186374 A	09-11-95
		EP 0757564 A	12-02-97
WO 9529713 A	09-11-95	AU 2399495 A	29-11-95
		EP 0757562 A	12-02-97
WO 9529712 A	09-11-95	AU 2369295 A	29-11-95
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		EP 0757563 A	12-02-97